AD_____

GRANT NUMBER DAMD17-96-1-6281

TITLE: Expression of Novel Steroid/Receptors in Mammary Development: Peroxisome Proliferator Activated Receptors

PRINCIPAL INVESTIGATOR: Jeffrey M. Gimble, M.D., Ph.D.

CONTRACTING ORGANIZATION: Oklahoma Medical Research Foundation
Oklahoma City, OK 73104

REPORT DATE: August 1997

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 1997	Annual (1 Aug 96 - 31 Jul 97)

4. TITLE AND SUBTITLE Expression of Novel Steroid/Receptors in Mammary Development: Peroxisome Proliferator Activated Receptors

5. FUNDING NUMBERS
DAMD17-96-1-6281

6. AUTHOR(S)
Jeffrey M. Gimble, M.D., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Oklahoma Medical Research Foundation
Oklahoma City, OK 73104

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

See introduction

14. SUBJECT TERMS Breast Cancer

15. NUMBER OF PAGES
15

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

___✓ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

___✓ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

____ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

____ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

____ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

PI - Signature _____ Date 8/13/97

TABLE OF CONTENTS

INTRODUCTION

Subject: This research will determine the expression pattern of a novel class of nuclear hormone receptors, the peroxisome proliferator activated receptors (PPARs), during mammary gland development in rodent models.

Purpose: The long-term goal of these studies is to determine if PPARs represent a potential target for the treatment of mammary tumors.

Scope of the research: The studies in this IDEA grant are limited to murine and rat models. They combine immunohistochemical and molecular biological approaches to examine the expression profile the different PPAR genes in mammary tissues from virgin, pregnant, and lactating mice and in rat mammary tumors induced by 7,12-dimethylbenzanthracene (DMBA).

Background of previous work: The epithelial cells of the mammary gland proliferate and mature in close proximity with the stromal adipocytes and fibroblasts. Stromal cells regulate the growth and development of the terminal end bud epithelial cells into alveolae and ducts through direct cell-cell interactions and the release of cytokines. The adipocytes of the mammary fat pad are critical for these epithelial events; transplanted mammary epithelial cells will only grow in the presence of adipose tissue [1,2]. This implies that mechanisms controlling adipogenesis impact on the physiology and pathology of the mammary gland in rodents and humans. Previous studies on bone marrow stromal cells and other pre-adipocyte models have demonstrated that the PPAR nuclear hormone receptors regulate adipocyte differentiation and the transcription of adipocyte-specific genes [3-5]. The PPAR ligands include the thiazolidinediones, used as oral anti-diabetic agents, and indomethacin, an anti-inflammatory drug [6-8]. The presence of these PPAR ligands induces pre-adipocyte stromal cells to differentiate into adipocytes *in vitro*. These compounds have potential relevance to mammary tumor suppression. Studies in a variety of rat mammary tumor models have demonstrated that indomethacin protected animals by reducing the incidence and size of tumors [9-12]. This action could not be attributed to the anti-inflammatory effects of indocmethacin since other anti-inflammatory drugs (salicylates, carprofen) had no protective effect in parallel experiments [11]. It is well established that the mechanisms underlying mammary development under physiologic and pathologic stimuli involve nuclear hormone receptors and their ligands. For example, the estrogen receptor is frequently mutated in human breast tumors and the estrogenic antagonist, tamoxifen, is commonly used as a chemotherapeutic agent following tumor resection. Based on these findings, we have undertaken studies to determine the expression profile of the PPAR genes in the rodent mammary gland in response to physiologic and pathologic stimuli.

BODY

Experimental methods, assumptions and procedures:

RNA analysis: Total RNA was prepared from 6-10 week old Balb/c female mouse mammary glands (virgin, pregnant, lactating or post-partum) according to published methods [13]. Northern blots were run with 10 μg per lane and hybridized with cDNA probes for PPARα, PPARβ/δ, PPARγ, and actin as previously described [6]. The relative levels of PPARγ1 and PPARγ2 were compared by quantitative PCR using isoform specific oligonucleotide primers listed below:

PPARγ1 Oligo #1F:TGTGTGACAGGAAACAGCTATGACCATG
PPARγ1 Oligo #1R:ATGCAGGTTCGTAAAACGACGGCCAGT
PPARγ1 Oligo #2F: TTTGAGCTCTTCTGACAGGACTGTGTGACAG
PPARγ1 Oligo #2R: TTTGAGACTCATAAGGTGGAGATGCAGGTTC
PPARγ2 Oligo #1F: TGAAACTCTGGAAACAGCTATGACCAT
PPARγ2 Oligo #1R: ATGCAGGTTCGTAAAAGCGACGGCCAGT
PPARγ2 Oligo #2F: TTTGAGCTCGCTGTTATGGGTGAAACTCTG
PPARγ2 Oligo #2R: TTTGAGCTCATAAGGTGGAGATGCAGGTTC

The first reaction was conducted with primer sets #1F and #1R with pBluescript SKII as template to generate a 246 bp product. These products then served as template for a second reaction using primer sets #2F and #2R to generate a final 287 bp product. Reactions were performed using a Perkin Elmer Cetus DNA Thermal Cycler for 35 cycles under the following conditions: 94°, 45 sec; 55°, 45 sec; 72°, 2 min. The final 287 bp products were purified on a non-denaturing acrylamide gel, their concentration determined by densitometry on an ethidium bromide stained analytical gel with a Stratagene Eagle Eye Video Imager system (San Diego CA), and used in competitive PCR reactions. Reactions were performed with serial ten-fold dilutions of competitor DNA fragment ranging from 25 pg to 0.025 fg of DNA in 50 μl volumes containing equal quantities of reverse transcribed virgin mammary gland cDNA (equivalent to 0.7 μg total RNA per reaction), 0.04 OD_{260} units of oligos #2F and #2R, 1 X reaction buffer (Promega, Madison WI), 1.5 mM $MgCl_2$ and 200 μM dNTP. Reactions were analyzed on 6% nondenaturing acrylamide gels stained with ethidium bromide.

Immunohistochemistry: Tissues from virgin, pregnant or lactating mice were fixed in phosphate buffered saline containing 10% formalin and paraffin embedded. Tissues were sectioned (5 μm thick) onto coated slides (Superfrost Plus, Fisher Scientific, Dallas TX), deparaffinized and the antigen unmasked by microwave treatment in 10 mM citrate buffer pH 6.0. Immunohistochemistry was performed with an affinity purified goat polyclonal antibody prepared against the C terminal peptide of the murine PPARγ protein [6]. Tissues were incbated with preimmune goat antibody or the affinity purified goat anti-murine PPARγ antibody for 30 minutes. Additional control experiments were performed with the anti-murine PPARγ antibody incubated in the presence of the multivalent antigenic peptide as a competitor. The primary goat antibodies were detected with an anti-goat avidin-biotin-horseradish peroxidase coupled reagent (Santa Cruz Biotechnology, Santa Cruz CA). Slides were counter-stained with methyl green or hematoxylin and eosin.

Treatment with DMBA: Virgin 55 day old Sprague Dawley rats (Harlan Sprague Dawley Labs, Indianapolis IN) were exposed to 20 mg DMBA (Sigma Chemcial Co, St. Louis MO) suspended in 1 ml of corn oil by gavage feeding [14]. After 16 weeks, mammary glands were harvested, fixed in phosphate buffered formalin and paraffin embedded prior to immunohistochemical analysis as described above. The histologic grade of tumors was assigned based on the criteria of Russo et al [15].

In situ mRNA hybridization: Fresh frozen murine mammary and adipose tissues were sectioned using a cryostat and hybridized with digoxigenin- labeled PPAR cDNA probes. Probes were localized with enzyme-tagged anti-digoxigenin antibodies.

Results and discussion:

The initial experiments have set out to determine how PPAR genes changed in response to physiologic events (Appendix, Page 11, Bottom). Based on northern blots, the mRNA levels of PPARα and PPARγ were abundant in virgin mice but declined during pregnancy and lactation. Levels increased in the involuted mammary glands of mice 2 weeks after the cessation of lactation (Nonlact-pp) but did not return completely to the virgin baseline. In contrast, the PPARβ/δ mRNA was constitutively epxressed at low levels under all conditions. The blots were hybridized with actin as a control; actin mRNA sizes are known to change during lactation in the mammary gland. Ethidium bromide staining confirmed that the ribosomal RNA was intact in all lanes and that loading was equivalent between lanes (not shown). Two explanations may explain the reduced levels of PPARα and PPARγ mRNA in the pregnant and lactating mammary gland. First, the adipocyte stromal cells may be the sole source of the PPAR mRNAs and, as adipocyte cell numbers in the mammary gland are reduced during the epithelial proliferation, the PPAR mRNA levels fall. Alternatively, both adipocyte in the stroma and epithelial cells may express PPAR mRNAs in the virgin mammary gland and these levels are reduced during pregnancy and lactation in response to hormonal cues.

The PPARγ mRNA can be found as two isoforms reflecting alternative splicing; PPARγ1 (1.8 kb) and PPARγ2 (2.1 kb) [16]. Since this difference in size cannot be easily distinguished on northern blots, quantitative PCR was used (Appendix, Page 11, Top). Analyses were conducted with cDNA prepared from virgin mammary glands in the presence of serial 10-fold dilutions of specific PPARγ1 or PPARγ2 competitors (Lanes A-G). When the concentration of native (N) and competitor (C) templates in each reaction were equal, their PCR products displayed equal signal intensity. These analyses determined that PPARγ2 mRNA awas 10-fold more abundant than PPARγ1 mRNA in the mammary gland. This is consistent with previous studies which have identified PPARγ2 as the predominant PPARγ isoform in adipose tissues [3].

This laboratory had previously developed an affinity purified, polyclonal goat antibody directed against the C-terminal peptide of the murine PPARγ protein [6]; unpublished studies have determined that the antibody will cross react with the PPARα but not PPARβ/δ proteins based. Histochemical analyses with this reagent examined the levels of PPARα and PPARγ immunoreactive proteins in the virgin, pregnant and lactating murine mammary gland (Appendix

Page 12). The pre-immune antibody (Pre) did not immunostain any of the murine tissues (Appendix, Page 12). Immunoreactivity with the anti-PPARγ (α-PPAR) was detected in the adipocytes of virgin and pregnant (day 8: P-8, day 17:P-17) mammary glands. However, by day 2 of lactation (L-2), PPAR immunoreactivity was absent in the mammary gland adipocytes. In contrast, the epithelial cells of the mammary gland contained immunoreactive protein under all physiologic conditions. The immunoreactive protein, detected in sections stained with antibody alone (indicated by "-"), was effectively competed away by co-incubation in the presence of the peptide antigen (indicated by "+") (Appendix, Page 13). The immunoreactive protein was most abundant in the cell cytoplasm. Presumably, transcription factors like the PPAR nuclear hormone receptors are found in the nucleus and fixation methods may account for the high levels observed in the cytoplasm. Additional analyses at higher magnification will be required to document nuclear localization of the immunoreactive protein. Studies are underway to examine the expression profile of the estrogen receptor in these tissues.

The anti-PPARγ antibody has been used to examine mammary tumor specimens from Sprague Dawley rats exposed to DMBA (Appendix, Page 13-14). Both benign (fibroadenoma:F) and malignant (papillary:P, comedo:C, tubular:T) tissue diagnoses have been observed. The level of immunoreactive PPAR proteins was reduced in the adipocytes and epithelial cells of the various tumors as compared to non-malignant tissues (N) obtained distal to the tumor specimens. Studies are underway on a panel of mammary tumor specimens using immunohistochemical methods.

Preliminary *in situ* hybridization studies with the PPAR cDNAs have not been successful. To date, studies have only been conducted with fresh frozen tissues and difficulties can be traced to the fatty nature of the mammary gland. It will be important to repeat these studies with paraffin embedded tissues. Paraffin sections will preserve the architecture better and will be more easily manipulated during the hybridization procedure.

Recommendations in relation to the Statement of Work:

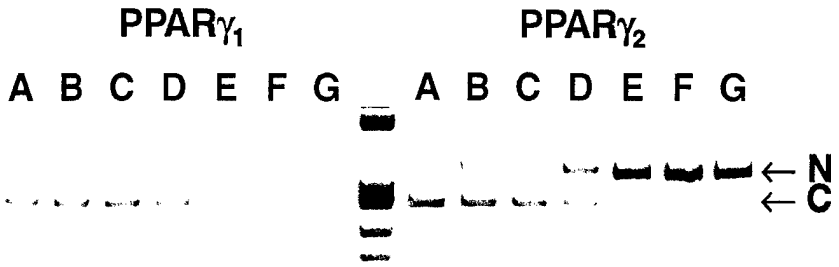
The project is progressing well within the time-frame outlined in the Statement of Work. Both murine and rat specimens have been collected and data analysis is underway. Further work will be required to improve the *in situ* hybridization technology. This will be a priority during the next year of the proposal.

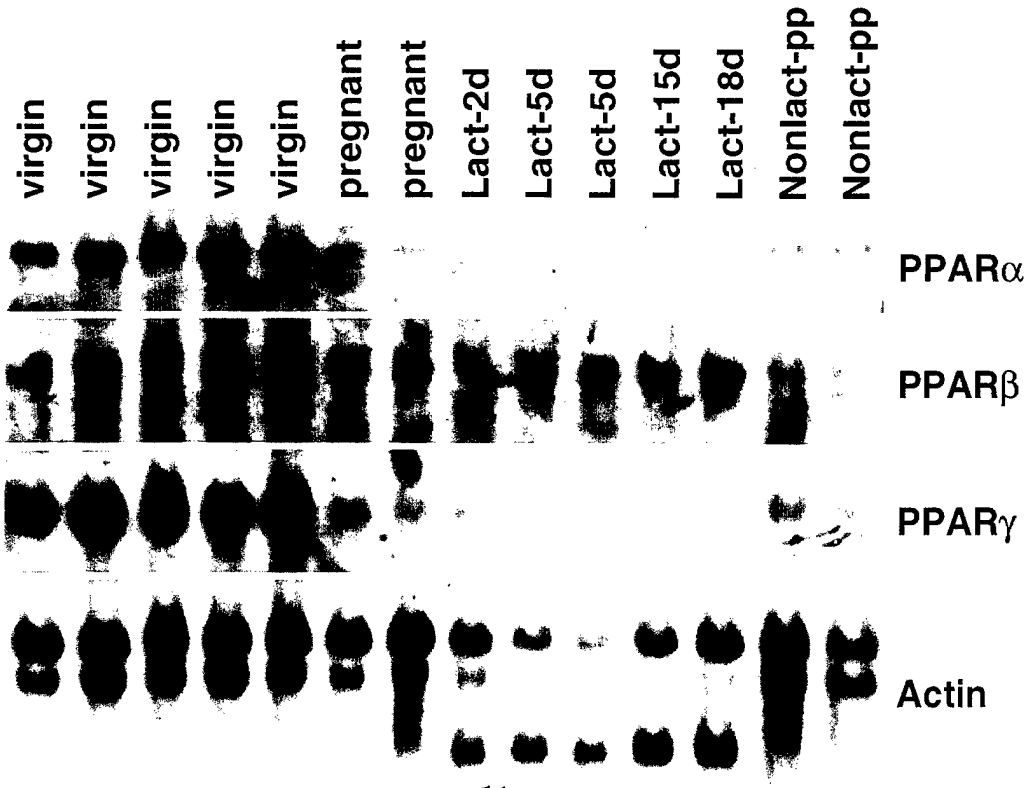
CONCLUSIONS

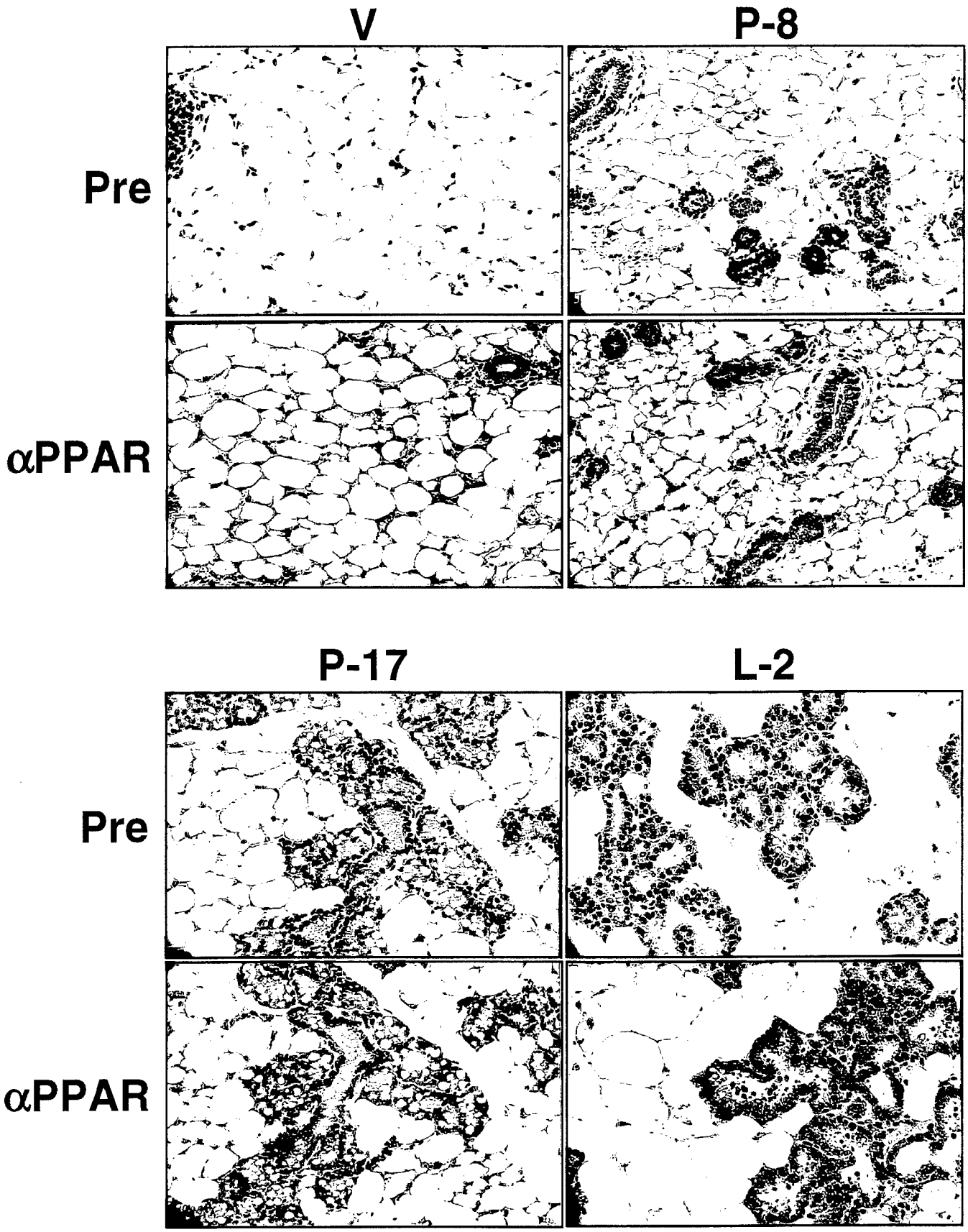
These preliminary studies document the presence of PPAR proteins in both the mammary stromal adipocytes and epithelial cells. The expression profile of the PPAR mRNA and proteins does not correlate directly. This pattern has been observed with other genes and may reflect altered stability of the PPAR mRNA and protein within adipocytes during pregnancy and lactation without changes in these levels in epithelial cells. These findings have important implications relating to mammary tumor development. Drugs binding to the PPAR-related nuclear hormone receptors, like the vitamin D and retinoic acid receptors, are known to inhibit mammary tumor progression in rodent models. With previous evidence that PPAR ligands may have a protective effect against mammary tumor development in animal models, this suggests that drugs binding to the PPAR proteins might have value as chemopreventive or chemotherapeutic agents in human breast cancer. As such, they could be valuable adjuvants to existing therapies for this disease.

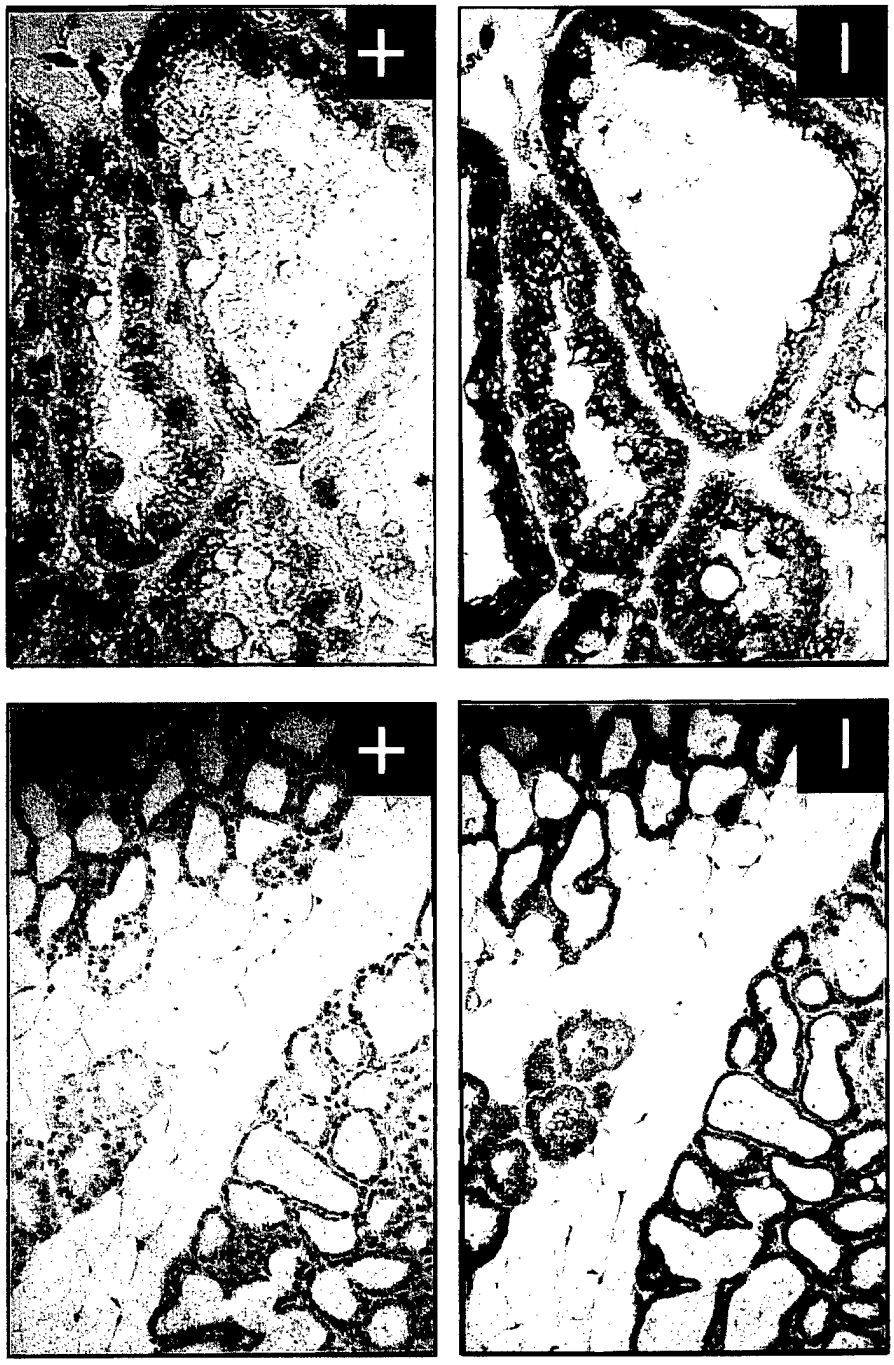
REFERENCES

1. Levine, J.F. and Stockdale, F.E. 1984. Exp. Cell Res. 151:112-122.
2. Levine, J.F. and Stockdale, F.E. 1985. J. Cell Biol. 100:1415-1422.
3. Tontonoz, P., Hu, E., Graves, R.A., Budavari, A. I. and Spiegelman, B.M. 1994. Genes & Dev. 8:1224-1234.
4. Forman, B.M., Tontonoz, P., Chen, J., Brun, R.P., Spiegelman, B.M. and Evans, R.M. 1995. Cell 83:803-812.
5. Kliewer, S.A., Lenhard, J.M., Willson, T.M., Patel, I., Morris, D.C. and Lehmann, J.M. 1995. Cell 83:813-820.
6. Gimble, J.M., Robinson, C.E., Wu, X., Kelly, K.A., Rodriguez, B.R., Kliewer, S.A, Lehmann, J.M. and Morris, D.C. 1996. Mol. Pharmacol. 50:1087-1094.
7. Lehmann, J.M., Moore, L.B., Smith-Oliver, T.A., Wilkison, W.O., Willson, T.M. and Kliewer, S.A. 1995. J. Biol. Chem. 270:12953-12956.
8. Lehmann, J.M., Lenhard, J.M., Oliver, B.B., Ringold, G.M. and Kliewer, S.A. 1997. J. Biol. Chem. 272:3406-3410.
9. Kollmorgen, G.M., King, M.M., Kosanke, S.D. and Do, C. 1983. Cancer Res. 43:4714-4719.
10. Carter, C.A., Milholland, R.J., Shea, W. and Ip, M.M. 1983. Cancer Res. 43:3559-3562.
11. Carter, C.A., Ip, M.M. and Ip, C. 1989. Carcinogenesis 10:1369-1374.
12. McCormick, D.L., Madigan, M.J. and Moon, R.C. 1985. Cancer Res. 45:1803-1808.
13. Chomczynski, P. and Sacchi, N. 1987. Anal. Biochem. 162:156-159.
14. Hollingsworth, A.B., Lerner, M.R., Lightfoot, S.A., Wilkerson, K.B., Hanas, J.S., McCay, P.B. and Brackett, D.J. In press, Breast Canc. Res. & Treatment.
15. Russo, J., Russo, I.H., Rogers, A.E., Van Zwieten, M.J. and Gusterson, B. in Pathology of Laboratory Animals: Vol. 1. Tumors of the Rat. V. Turosov, U. Mohr (eds.). Pgs. 47-78.
16. Zhu, Y., Qi, C., Korenberg, J.R., Chen, X.-N., Noya, D., Rao, N.S. and Reddy, J.K. 1995. Proc. Natl. Acad. Sci. USA 92:7921-7925.


PPARγ1
PPARγ2
A B C D E F G
A B C D E F G
N
C


virgin
virgin
virgin
virgin
virgin
pregnant
pregnant
Lact-2d
Lact-5d
Lact-5d
Lact-15d
Lact-18d
Nonlact-pp
Nonlact-pp
PPARα
PPARβ
PPARγ
Actin


V
P-8
Pre
αPPAR
P-17
L-2
Pre
αPPAR


+
I
+
I

N
C
T
Pre
αPPAR

N
F
P
Pre
αPPAR